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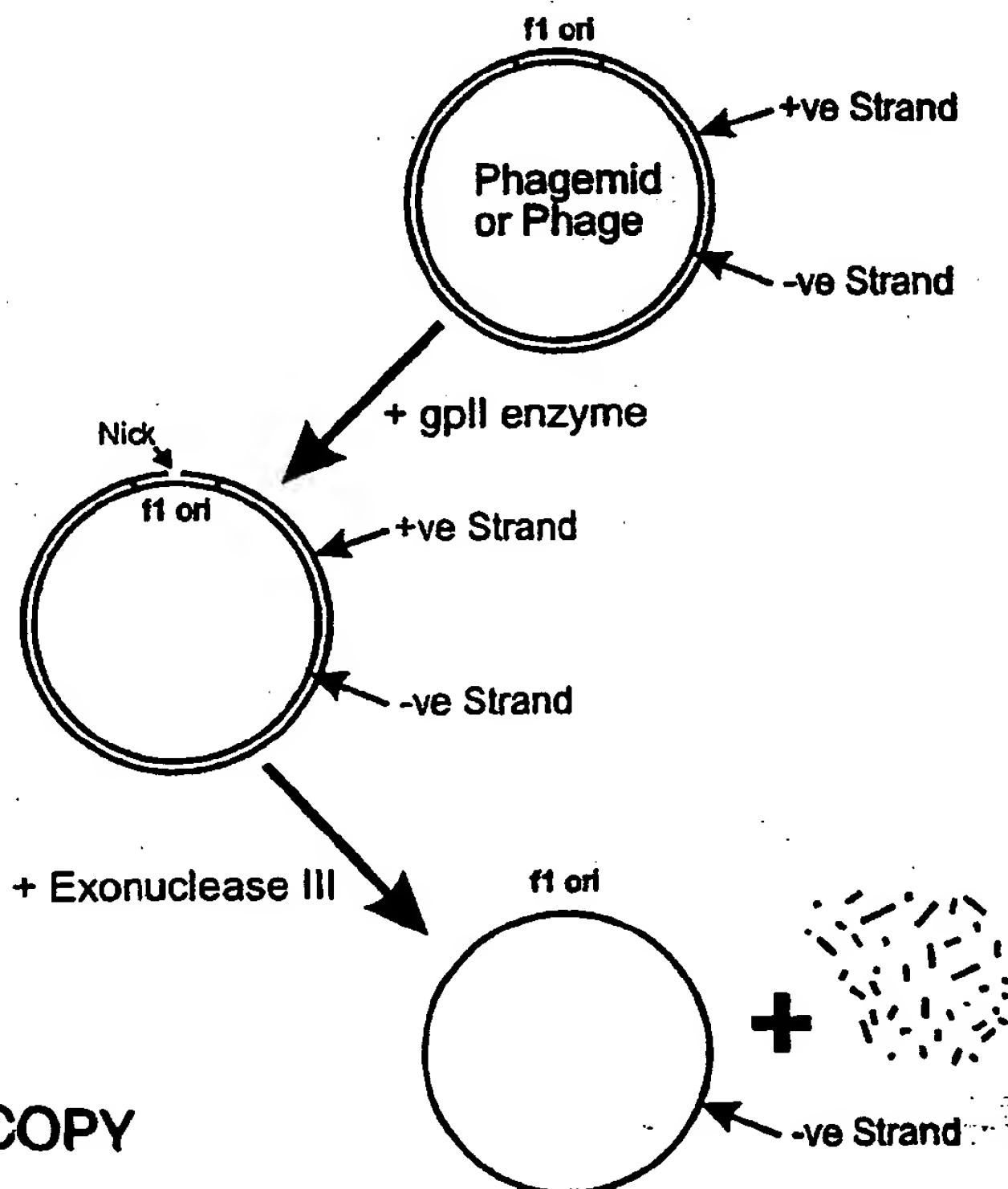
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(54) Title: *IN VITRO* METHOD FOR CIRCULAR SINGLE-STRANDED DNA

(57) Abstract

The invention relates to an *in vitro* method for preparing circular single-stranded DNA from circular double-stranded DNA comprising the steps of: (i) introducing a nick into a specific strand of the dsDNA; and (ii) removing the nicked strand to produce single-stranded DNA. The invention also provides a kit of components for use in the method of the invention.

Preparation of Single-Stranded DNA



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IN VITRO METHOD FOR CIRCULAR SINGLE-STRANDED DNA.

The present invention relates to an *in vitro* method of preparing circular single stranded ("ss") DNA from circular double stranded ("ds")DNA.

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DNA sequencing and site-directed mutagenesis are two of the key techniques in modern molecular biology. DNA sequencing is the central technique of the Human Genome (HUGO) Project underway in the EC, USA and Japan. This is a very long term project which has necessitated great advancements in the field of automated sequencing in order to succeed over a reasonable time-span. However, techniques in ssDNA preparation required before optimal DNA sequencing can take place have not developed at the same pace. Single-stranded DNA is used in manual and automated sequencing techniques involving the chain termination method such as that developed by Sanger.

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In order to study the function of proteins it often necessary to alter their structure specifically. This can be achieved by site-directed mutagenesis which allows the effects of very slight changes to be measured and the original functions deduced.

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The most widespread and effective ways of performing the above techniques are dependent upon the production of single stranded DNA molecules. At present single-stranded DNA (ssDNA) is generated by the somewhat cumbersome technique of "single-stranded rescue." This involves cloning the DNA of interest into either a filamentous bacteriophage vector, or a plasmid containing a replication origin from a filamentous bacteriophage (such plasmids are termed "phagemids"). An example of a filamentous bacteriophage vector is M13mp18 and of a phagemid is pBluescript™.

30

Bacteria carrying filamentous bacteriophage produce virus particles containing ssDNA that can be recovered from the growth medium. In the case of phagemids, it is necessary to infect the host bacterium with a suitable filamentous 'phage in order to initiate ssDNA production. Once again, ssDNA carrying the cloned insert can be purified from the bacterial growth medium.

Both of the above *in vivo* techniques have their associated problems. For example,

DNA cloned into vectors based on filamentous bacteriophage tends to be unstable, deletions often occur and, in general, the larger the cloned insert the worse this problem becomes. Frequently, filamentous bacteriophage vectors are biased towards a particular orientation of the foreign DNA insert and this can be very frustrating for researchers who are trying to sequence both strands of the inserted DNA. Phagemids overcome some of the problems associated with the filamentous bacteriophage as a result of the following properties:

- they can carry many of the features of normal plasmids (expression signals etc);
- cloned inserts are usually inherited stably;
- they are sufficiently small that fragments of DNA up to 10 kb long can be carried;
- with phagemids there is no need to perform time-consuming subcloning to bacteriophage-based vectors.

However, phagemids suffer from the considerable disadvantage that they give lower and less reproducible yields of ssDNA than filamentous 'phage vectors. Consistent yields can also be a problem with this method and it may be necessary to experiment with different helper 'phage to obtain consistent results.

Problems encountered in the purification of ssDNA from bacterial culture supernatants are common to both vector systems. These include:

- contamination with non-recombinant phage DNA;
- contamination with bacterial DNA and RNA;

- DNA is subject to shear forces during purification which leads to breakage.
- carry-over of chemicals, notably polyethylene glycol and phenol, from the purification procedure into further sequencing or mutagenesis reactions.

5 These substances are undesirable because they interfere with subsequent manipulation of the DNA.

- There is a requirement for the preparation of sterile culture media, maintenance of helper phage stocks, growth of individual clones, infection with helper phage, isolation of phage, and purification of single-stranded DNA from these isolates. This routine and repetitive work takes up much valuable technical time and resources.

15 However, the main disadvantages of present *in vivo* single-stranded rescue techniques are that they are slow, labour intensive and not amenable to automation.

The quest for automation has led, in the case of DNA sequencing, to the development of techniques which use double-stranded DNA as a template (see for example Current
20 Protocols in Molecular Biology (1990) Eds. Ausubel, F. *et al* Greene Associates/Wiley Interscience, New York; and Molecular Cloning: A Laboratory Manual (1989) Eds. Sambrook, J.*et al* ,Cold Spring Harbour Laboratory, Cold Spring Harbour). While there have been tremendous improvements in this technology over recent years, the following problems still exist:

25

- Results are very dependent on the quality of the plasmid preparation used as template. Extra care must therefore be taken at the plasmid purification stage and this means that the saving in time and effort over single-strand rescue techniques is reduced.
- The quality of the sequencing gels is never as good as that obtained with ssDNA. The technique is particularly prone to error with certain "difficult" sequences, for

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example GC-rich sequences which have the potential to form unique secondary structures. This not only leads to time-consuming revisions of experimental strategy, for example the region might have to be re-sequenced using an alternative polymerase or the experimenter may have to revert to the use of a single-stranded template, but is also a limiting factor in the automation of sequence-gel reading. This is because the available software in automated gel-readers is generally not sophisticated enough to sense when a "difficult" region of DNA is being sequenced.

There is a great need for the development of an *in vitro* method for the preparation of ssDNA which would circumvent many of the problems associated with conventional *in vivo* techniques and be amenable to automation. This last feature would satisfy the urgent needs of researchers involved in sequencing large genomes.

According to the invention there is provided an *in vitro* method for preparing circular single-stranded DNA from circular double-stranded DNA comprising the steps of:

(i) introducing a nick into a specific strand of the dsDNA; and

(ii) removing the nicked strand to produce single-stranded DNA.

By "specific strand" we mean either the plus or minus strand of the dsDNA. Hence the ssDNA produced is either the plus or minus strand, not a mixture of the two.

Preferably, the ssDNA produced by the method of the invention is intact, that is to say it comprises the sequence of one entire strand of the original dsDNA.

The circular single-stranded DNA produced by the method of the invention is suitable for use in a range of applications including DNA sequencing and site-directed mutagenesis experiments. The method of the invention is particularly advantageous in that it reduces the labour time and cost spent on single-stranded DNA preparation by over 50% compared to current technology.

The ability to produce circular ssDNA from circular dsDNA is a particular advantage since most genetic manipulation techniques produce dsDNA in circular form, for example, as a plasmid. Furthermore it is advantageous to sequence both strands of a given DNA fragment to ensure accuracy of the deduced sequence. The method of the present invention typically produces negative strand single-stranded DNA, whereas current *in vivo* single-stranded rescue techniques prepare the positive strand. Therefore by combining the method of the present invention with a current technique it is possible to prepare and sequence both strands from a clone without the necessity of preparing another subclone. This results in a considerable saving in time.

Advantageously, the specific strand is nicked enzymatically. Preferably the strand is nicked by an enzyme from the family of filamentous bacteriophages of *Escherichia coli* including M13, fl, and fd, or Pfl from *Pseudomonas* species. Other suitable enzymes may come from plasmids which are capable of conjugal transfer where there are systems which produce single-stranded DNA prior to the transfer step. An example of such plasmids are the plasmid family incP (see Guiney, D. & Lanka, E. in Promiscuous plasmids of Gram-Negative Bacteria (1989), Ed. C. Thomas, Academic Press, London.) Strand specific nicking can also be achieved by DNA topoisomerases and DNA gyrases (see for example Reece, R. & Maxwell, A. (1991) Crit.Rev.Biochem. Mol. Biol. 26 335-375).

A particularly preferred nicking enzyme is the gpII protein from the phage M13 which nicks at the f1 ori. The nicking site of fl, fd, and M13 is between the T and A of TTAA in the positive strand sequence of TCCACGTTCTTT↓AATAGTGGACT.

The gpII proteins of fl, fd and M13 display almost complete homology with each other (van Wezenbeek, P. *et al.* (1980) *Gene* 11 129-148). All three enzymes, which are also referred to in the literature as "gene II protein", can be used to advantage because a wide range of phagemids with f1 origins of replication, which are nicked by the gpII protein, are already in widespread use. Examples of these phagemids are the prokaryotic vectors pGEMTM and pBluescriptTM, as well as eukaryotic vectors such as the *E. coli* shuttle vectors pSVK11 and pSVK111.

Where the nicking enzyme is gpII, the positive strand of the dsDNA is nicked in the presence of Mg²⁺ or Ca²⁺. Thus it is possible to select for the removal of a particular

strand.

5 Preferably, the nicked strand is removed by enzymatic degradation, preferably using an exonuclease, with exonuclease III from *E. coli* being particularly suitable. Examples of other enzymes which may be used to remove the nicked strand include T7 gene 6 exonuclease, T5 D15 exonuclease and λ exonuclease. gpII is known to leave a 3'-OH for exonuclease III and a 5' phosphate residue for T7 gene 6 exonuclease, T5 D15 exonuclease or λ exonuclease (Meyer, T., Geider, K., Kurz, C. & Schaller, H. (1979) *Nature* 278 p365). Examples of the use of T7 gene 6 exonuclease and exonuclease III can
10 be found in Straus, N. & Zagursky, R. (1991) *BioTechniques* 10 p376 and for λ exonuclease in Higuchi, R & Ochman, H. (1989) *Nucleic Acids Res.* 17 p5865. An example of the use of T5 D15 exonuclease is in Sayers, J. & Eckstein, F. (1991) *Nucleic Acids Research* 19 4127-4132.

15 Advantageously, the steps (i) and (ii) of the method of the invention are performed simultaneously. However, the method may include a further step of removing the nicking enzyme between steps (i) and (ii).

20 This can be achieved using a conventional purification techniques, for example DNA purification columns, phenol extraction, immobilised matrices and glassmilk purification.

25 It is preferred that the method of the invention includes a further step of removing the unwanted degradation products so that they do not interfere with subsequent uses of the ssDNA product. This can be achieved by means of a conventional DNA purification column, isopropanol or ethanol precipitation with a salt such as ammonium acetate or phenol extraction followed by isopropanol/ethanol precipitation.

30 According to another aspect of the invention there is provided a kit comprising the components necessary for carrying out the method in the form of an enzyme for introducing a nick into a specific strand of dsDNA and an enzyme for removing the nicked strand. Preferably, the nicking enzyme is gpII and the enzyme for removing the nicked strand is exonuclease III.

According to a further aspect of the invention there is provided a method of DNA sequencing or site-directed mutagenesis comprising preparing single stranded DNA by the method of the invention.

5 A method of preparing ssDNA in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings, Figures 1 and 2, in which:

10 Fig.1 is a schematic diagram illustrating the method of the invention; and

Fig.2 is a schematic diagram illustrating alternative embodiments of the method of the invention.

I. Method Summary

15

As shown in Fig. 1, and in the left hand limb of Fig. 2 a single nick is introduced into the positive strand of the f1 origin of replication of a supercoiled plasmid DNA (represented by concentric circles in Fig.1 for the sake of simplicity) by the site specific gpII enzyme. The nicked plasmid is then used as a substrate for exonuclease III to produce a single- stranded negative strand suitable for use as a substrate for DNA sequencing or DNA mutagenesis. In the alternative method according to the righthand limb of Fig.2, the nicking enzyme gpII and the degrading enzyme exonuclease III can be added simultaneously to the dsDNA to be processed into ssDNA.

25

II. gpII plasmid cleavage reaction producing nicked positive strand

30 A. Substrate: The plasmid preparation should contain a high proportion of supercoiled DNA, as relaxed circles or linear DNA will not be nicked so efficiently by the gpII enzyme. Commonly used sources for the DNA are the phagemids which contain a f1 origin of replication in both orientations in addition to a double-stranded replication origin such as those sold under the trade marks pBluescript™ or pGEM™. The dual orientation of the f1 origin in these vectors allows the preparation of both strands of

a cloned sequence by this method.

5 B. Site specificity: the gpII enzyme recognises a specific DNA sequence. Meyer T., *et al* (1979) in *Nature* 278 365-367 describe the cleavage site of bacteriophage fd gene II protein and, since fd, M13 and f1 display almost complete homology with each other, the gpII protein from M13 is known to recognise the same sequence.

10 C. Supercoiling specificity: Under strand-specific nicking reaction conditions, gpII will only nick supercoiled DNA. Relaxed plasmid DNA or single-stranded DNA are not substrates for the gpII enzyme.

15 D. Strand specificity: If Mg^{2+} or Ca^{2+} is used in the reaction conditions, only the positive strand of supercoiled DNA is cleaved. Thus nicking of the positive strand of DNA can be selected for.

E. Cleavage reaction: the gpII enzyme introduces a single-strand break in the positive strand of M13 or phagemids containing a M13, f1 or fd ori sequence between CTTT↓AAT to leave 5'PO₄ and 3'OH ends.

20 F. Reaction conditions: Suitable reaction conditions for gpII cleavage are 5-20 units of gpII enzyme with 2-4 picomols of supercoiled plasmid in a 20μl volume of 25-50 mM Tris-HCl, pH 6.5-8.5, preferably 8.0, 0-120mM NaCl or KCl, 5mM DTT (dithiothreitol), 0.5-10mM MgCl₂ (or 0.5-10 mM CaCl₂), preferably 0.5mM. Incubate at a temperature of from 30-44°C, for 10-60 minutes, preferably for 15 minutes.

25 The reaction can be followed on agarose gels by the change in mobility of the plasmid as it is converted from its supercoiled to relaxed forms. The gpII enzyme has a requirement for divalent metal ions and the other components help to stabilise the cleavage or nicking reaction. There are a number of alternative components which act to
30 stabilise the reaction and these are within the knowledge of a skilled worker.

III. Preparation for Treatment with Exonuclease III

A. The reaction time from step I.(F) is halted by the addition of 40 μ l of 100 mM Tris-HCl, pH 7.4, 20mM EDTA, 1M NaCl.

5 B. The reaction mixture is then extracted twice with an equal volume of phenol/chloroform/isoamylalcohol (50:48:2) and once with an equal volume of chloroform/isoamylalcohol (24:1), centrifuging in a microcentrifuge for 5 minutes between each extraction and removing the top aqueous layer.

10 C. The DNA is precipitated with two volumes of cold ethanol, centrifuged in a microcentrifuge for 10 minutes, the supernatant is discarded, the pellet is washed gently with cold 70% (v/v) ethanol, re-centrifuged, the supernatant is discarded and the pellet is resuspended in 18 μ l of 10mM Tris-HCl, pH8.0, 1mM EDTA.

15 Alternatively, heat inactivation, no treatment, a conventional DNA purification column, and isopropanol or ethanol precipitation with a salt such as ammonium acetate can be used.

In another embodiment, the preparation for treatment with exonuclease III, is omitted and it is preferred that the gpII and exonuclease III enzymes are used simultaneously at the start of the method. Preferred reaction conditions are 25-50mM Tris-HCl pH6.5-
20 8.5, preferably 8.0, 0-120mM NaCl or KCl, 1mM MgCl₂, 5mM DTT, 5-20 units gpII enzyme, 10-120 units exonuclease III/ μ g DNA, 2-4 picomoles of supercoiled plasmid in a 20 μ l reaction volume.

IV. EXONUCLEASE III DIGESTION OF NICKED STRAND

25

A. 2 μ l of 10x exonuclease III buffer is added to the DNA recovered in step III.C (10x exonuclease III buffer is 250-500mM Tris-HCl, pH 8.0, 0-1.2M NaCl, 10 mM MgCl₂). 10U exonuclease III is added per μ g DNA.

30

B. The reaction mixture is incubated at 37°C for ten minutes per kilobase.

C. The reaction is stopped by adding 2 μ l 0.5M EDTA and the reaction mixture is heated to 75°C for 10 minutes.

D. Residual nucleotides and oligonucleotides are removed, for example by passing through a Sephadex™ G50 column or an immobilized matrix. The DNA is now ready for use as a single-stranded negative strand template for DNA sequencing or mutagenesis.

5 The following additional method steps can also be included to improve the purity of the ssDNA produced:

E. Recover the DNA from step D and add potassium acetate, pH 5.2 to 300mM.

10 F. Precipitate with ethanol as described in step III.C, resuspend in 10μl TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA).

15 By using M13 derived vectors that allow the sequence of interest to be cloned in either orientation, it is possible to sequence both strands during the above negative strand preparation process.

In summary, the present invention overcomes the problems of conventional *in vivo* techniques by providing a simple and efficient *in vitro* method of preparing circular ssDNA from a circular dsDNA template, which method is amenable to automation.

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CLAIMS

- 1 An *in vitro* method for preparing circular single-stranded DNA from circular
double-stranded DNA comprising the steps of:
- 5 (i) introducing a nick into a specific strand of the dsDNA; and
- (ii) removing the so nicked strand to produce single-stranded DNA.
- 10 2 A method according to claim 1 in which the specific strand is the positive or
negative strand of the dsDNA.
- 3 A method according to claim 1, or 2 in which the ssDNA produced is either of
the positive or negative strands of the original dsDNA.
- 15 4 A method according to any preceding claim in which the specific strand is nicked
enzymatically.
- 5 A method according to claim 4 in which the strand is nicked by an enzyme from
20 the family of filamentous bacteriophages of *Escherichia coli*, or from
Pseudomonas species or from plasmids which are capable of conjugal transfer, or
by DNA topoisomerases and DNA gyrases.
- 6 A method according to claim 5 in which the nicking enzyme is the gpII protein
25 from the phage M13.
- 7 A method according to any preceding claim in which the nicked strand is
removed by enzymatic degradation.
- 30 8 A method according to claim 7 in which the enzymatic degradation is performed
using an exonuclease.
- 9 A method according to claim 8 in which the exonuclease is exonuclease III from

E. coli, T7 gene 6 exonuclease, T5 D15 exonuclease or λ exonuclease.

- 10 A method according to any preceding claim in which steps (i) and (ii) are performed simultaneously.
- 5
- 11 A method according to any one of claims 1-9 including the step of removing the nicking enzyme between steps (i) and (ii).
- 12 A method according to any preceding claim including the further step of removing unwanted DNA degradation products from the ssDNA.
- 10
- 13 A method according to claim 12 in which potassium acetate is added to the DNA, which is then precipitated with ethanol to improve the purity of the ssDNA.
- 15
- 14 A method according to any preceding claim in which the dsDNA is derived from a phagemid.
- 15 A kit comprising an enzyme for introducing a nick into a specific strand of circular dsDNA and an enzyme for removing the nicked strand to produce ssDNA.
- 20
- 16 A kit according to claim 15 in which the nicking enzyme is gpII and the enzyme for removing the nicked strand is exonuclease III.
- 25
- 17 A method of DNA sequencing or site-directed mutagenesis comprising preparing single stranded DNA by a method according to any preceding claim.

Preparation of Single-Stranded DNA

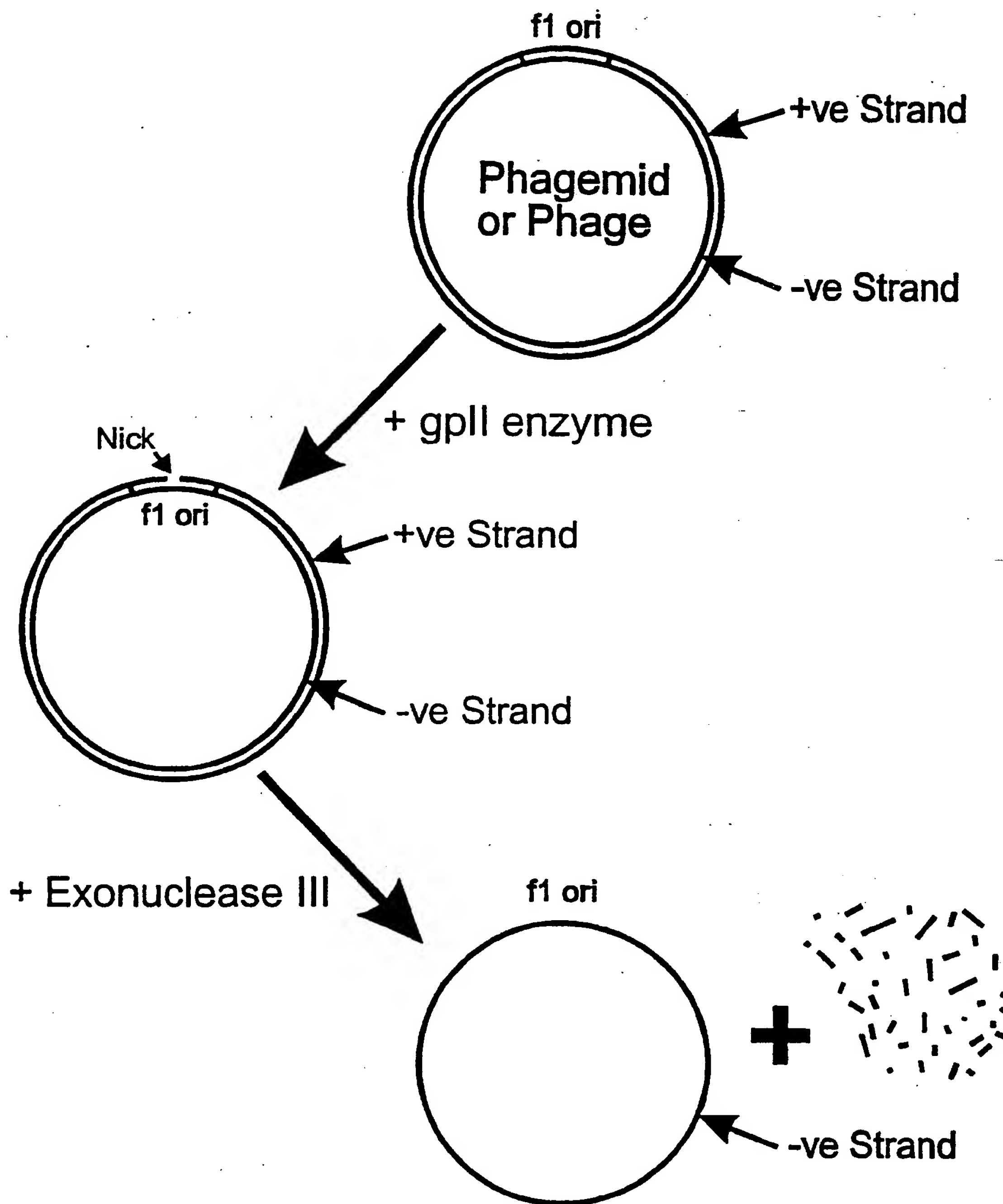


Figure 1

Single-Stranded DNA Preparation Alternative Protocols

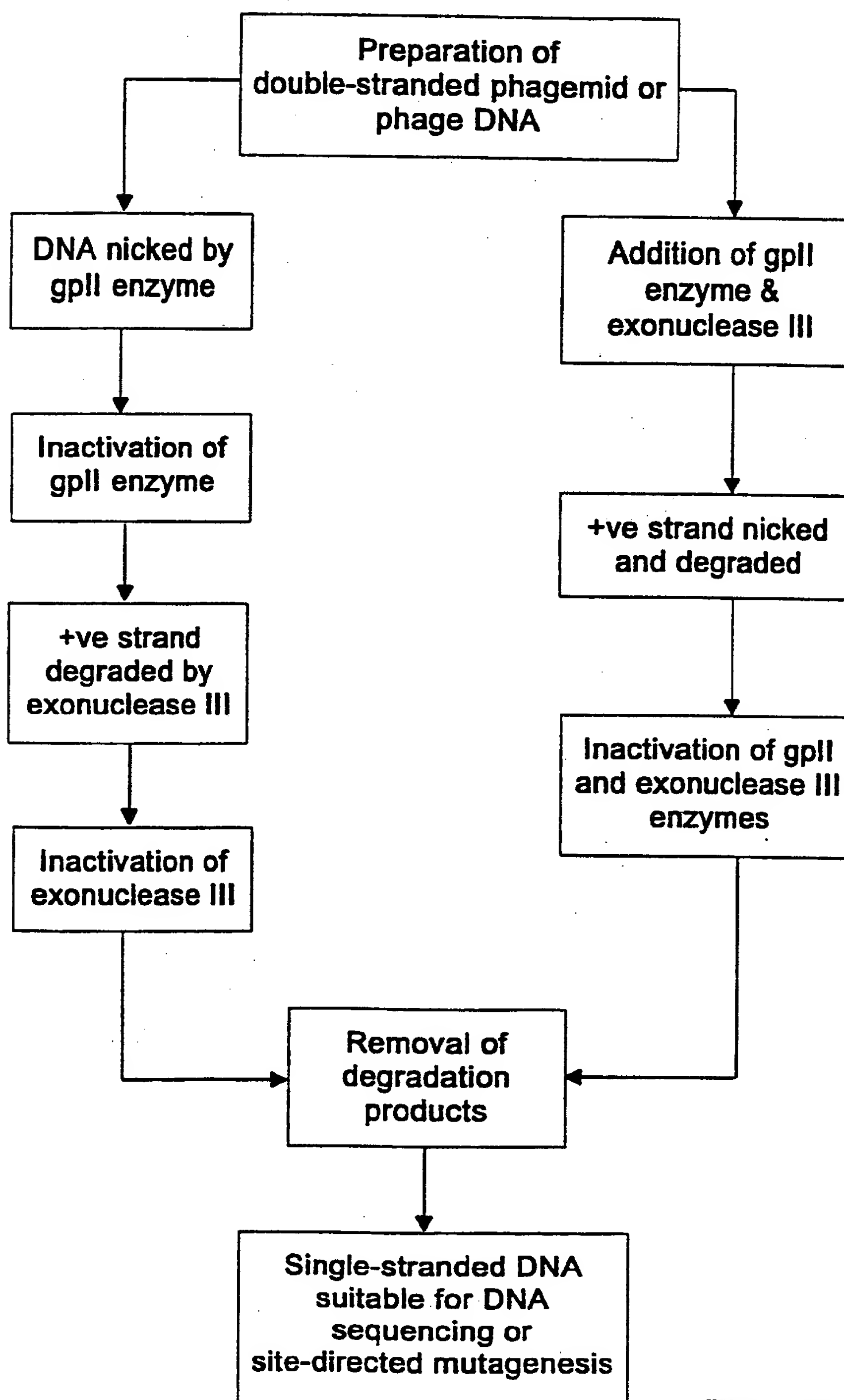


Figure 2

INTERNATIONAL SEARCH REPORT

International Application No

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 06645 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 16 May 1991	1-4,7,8,17
Y	see page 9, line 34 - line 36	5,6,12-14
	see page 13, line 1 - page 15, line 18	
X	NUCL. ACIDS RES., vol.16, no.3, 11 February 1988, IRL PRESS, OXFORD, ENGLAND; pages 791 - 802	1-4,7-9
	J.R. SAYERS ET AL. '5'-3' Exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis'	
Y	see page 795, line 1 - page 802, line 17; figures 2,3	5,6,12-14
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☒ Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>NUCL. ACIDS RES., vol.16, no.3, 11 February 1988, IRL PRESS, OXFORD, ENGLAND; pages 803 - 814 J.R. SAYERS ET AL. 'Strand specific cleavage of phosphorothioate-containing DNA by reaction with restriction endonucleases in the presence of ethidium bromide' see page 807, line 19 - page 813, line 8 ---</p>	<p>1-9, 12-14</p>
Y	<p>NUCL. ACIDS RES., vol.19, no.15, 11 August 1991, IRL PRESS, OXFORD, ENGLAND; pages 4127 - 4132 J.R. SAYERS AND F. ECKSTEIN 'A single-strand specific endonuclease activity copurifies with overexpressed T5 D15 exonuclease' cited in the application see page 4129, right column, paragraph 4 - page 4131, right column, line 53; figures 1-5; table I ---</p>	<p>1-9, 12-14</p>
Y	<p>NATURE, vol.278, 22 March 1979, MACMILLAN JOURNALS LTD., LONDON, UK; pages 365 - 367 T.F. MEYER ET AL. 'Cleavage site of bacteriophage fd geneII-protein in the origin of viral strand replication' cited in the application the whole document ---</p>	<p>5-7</p>
Y	<p>WO,A,90 14416 (GENETICS INSTITUTE, INC.) 29 November 1990 see page 1, line 24 - page 2, line 7 see page 11, line 1 - page 14, line 21; claims 1-18 ---</p>	<p>12,13</p>
Y	<p>WO,A,91 12341 (FRED HUTCHINSON CANCER RESEARCH CENTER) 22 August 1991 see page 5, line 6 - line 15; figures 1,2 -----</p>	<p>14</p>

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 94/02172

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9106645	16-05-91	DE-C- 3936258	25-04-91
WO-A-9014416	29-11-90	US-A- 5047345	10-09-91
		DE-D- 68914689	19-05-94
		DE-T- 68914689	27-10-94
		EP-A- 0473575	11-03-92
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